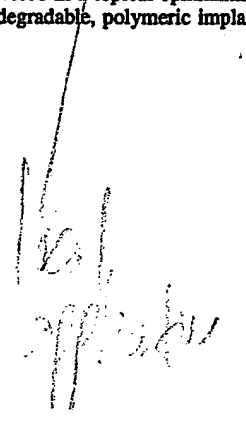


PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶: A61K 38/51	A1	(11) International Publication Number: WO 95/13830 (43) International Publication Date: 26 May 1995 (26.05.95)
(21) International Application Number: PCT/US94/13343 (22) International Filing Date: 17 November 1994 (17.11.94) (30) Priority Data: 153,873 17 November 1993 (17.11.93) US (71) Applicants: MASSACHUSETTS INSTITUTE OF TECHNOLOGY [US/US]; 77 Massachusetts Avenue, Cambridge, MA 02139 (US). CHILDREN'S MEDICAL CENTER CORPORATION [US/US]; 55 Shattuck Street, Boston, MA 02115 (US). (72) Inventors: SASISEKHARAN, Ramnath; 118 Ridge Street, Arlington, MA 02174 (US). MOSES, Marsha, A.; 64 Dean Road, Brookline, MA 02146 (US). NUGENT, Matthew, A.; 6 Jeffrey Circle, Bedford, MA 01730 (US). COONEY, Charles, L.; 35 Chestnut Place, Brookline, MA 02146 (US). LANGER, Robert, S.; 77 Lombard Street, Newton, MA 02158 (US). (74) Agents: PABST, Patrea, L. et al.; Arnall Golden & Gregory, 2800 One Atlantic Center, 1201 West Peachtree Street, Atlanta, GA 30309-3400 (US).		(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: METHOD FOR INHIBITING ANGIOGENESIS USING HEPARINASE (57) Abstract Pharmaceutical compositions for delivering an effective dose to a desired site of a heparinase. These compositions are based on the discovery that heparinase alone can inhibit angiogenesis. The effective dosage is dependent not only on the heparinase, but also on the method and means of delivery, which can be localized or systemic. For example, in some applications, as in the treatment of psoriasis or diabetic retinopathy, the inhibitor is delivered in a topical ophthalmic carrier. In other applications, as in the treatment of solid tumors, the inhibitor is delivered by means of a biodegradable, polymeric implant. 		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

METHOD FOR INHIBITING ANGIOGENESIS USING HEPARINASE

Background of the Invention

The United States government has rights in this invention by virtue of National Institutes of Health GM25810 to Robert S. Langer and a National Science Foundation grant to Charles Cooney.

5 Angiogenesis is a fundamental process by which new blood vessels are formed, as reviewed, for example, by Folkman and Shing, J. Biol. Chem. 267 (16), 10931-10934 (1992). It is essential in reproduction, development and wound repair, as
10 discussed by Folkman, et al., Science 243, 1490-1493 (1989). Under these conditions, angiogenesis is highly regulated, so that it is turned on only as necessary, usually for brief periods of days, then completely inhibited.

15 Unregulated neovascularization is integral to the pathogenesis of various diseases including arthritides, primary and metastatic tumors and diseases of the eye such as diabetic retinopathy, age-related macular degeneration, branch retinal
20 vein occlusion, retinopathy of prematurity, and neovascular glaucoma. There is now increasing evidence between the neovascular process and prognosis of diseases (Weidner, et al, (1991) N. Engl. J. Med. 324, 1; Bosari, et al, (1992) Human
25 Pathol. 23, 755). Regulation of angiogenesis would be a critical means for intervention in a multiplicity of angiogenesis dependent diseases. For example, promotion of angiogenesis has been shown to enhance healing of wounds (Klagsbrun and
30 Vlodavsky, (1988) *In Growth Factors and Other Aspects of Wound Healing, Biological and Clinical Implications*, New York, Alan R. Liss), and peptic ulcer, while angiogenesis inhibition in in vitro and in vitro models has been shown to significantly
35 affect certain diseases processes, including tumors (Moses and Langer, (1991) Biotechnology 9, 630).

A number of serious diseases are dominated by abnormal neovascularization including solid tumor growth and metastases, some types of eye disorders, and rheumatoid arthritis, reviewed by Auerbach, et al., J. Microvasc. Res. 29, 401-411 (1985); Folkman, Advances in Cancer Research, eds. Klein and Weinhouse, pp. 175-203 (Academic Press, New York 1985); Patz, Am. J. Ophthalmol. 94, 715-743 (1982); and Folkman, et al., Science 221, 719-725 (1983). In arthritis, new capillary blood vessels invade the joint and destroy cartilage. Tumor growth and metastasis are angiogenesis-dependent. A tumor must continuously stimulate the growth of new capillary blood vessels for the tumor itself to grow. In diabetes, new capillaries invade the vitreous, bleed, and cause blindness. There are a number of eye diseases, many of which lead to blindness, in which ocular neovascularization occurs in response to the diseased state. These ocular disorders include diabetic retinopathy, neovascular glaucoma, inflammatory diseases and ocular tumors (e.g. retinoblastoma). There are a number of other eye diseases which are also associated with neovascularization, including retrolental fibroplasia, uveitis, approximately twenty eye diseases associated with choroidal neovascularization and approximately forty eye diseases which are associated with iris neovascularization. The current treatment of these diseases is inadequate, especially once neovascularization has occurred, and blindness often results.

Capillary blood vessels consist of endothelial cells and pericytes. These two cell types carry all of the genetic information to form tubes, branches and whole capillary networks. Specific angiogenic molecules can initiate this

process. Specific inhibitory molecules can stop it. These molecules with opposing function appear to be continuously acting in concert to maintain a stable microvasculature in which endothelial cell turnover is thousands of days. However, the same endothelial cells can undergo rapid proliferation, i.e. less than five days, during burst of angiogenesis, for example, during wound healing.

A number of proteases have been implicated as key factors in angiogenesis. See, for example, Mignatti, et al., Cell 47, 487-498 (1986) and Rifkin, et al., Acta. Biol. Med. Germ. 40, 1259-1263 (1981), who suggest several enzymes in a proteolytic cascade, including plasminogen activator and collagenase, must be inhibited in order to inhibit angiogenesis.

The angiogenic process is known to proceed in defined steps (Folkman, J., (1990) Cancer Res. 46, 467). Key components of the angiogenic process are the degradation of the basement membrane, the migration and proliferation of capillary endothelial cell (EC) and the formation of three dimensional capillary tubes. The normal vascular turnover is rather low: the doubling time for capillary endothelium is from 50-20,000 days, but it is two to thirteen days for tumor capillary endothelium. The current understanding of the sequence of events leading to angiogenesis is that a cytokine capable of stimulating endothelial cell proliferation, such as fibroblast growth factor (FGF), causes release of collagenase or plasminogen activator which, in turn, degrade the basement membrane of the parent venule to facilitate in the migration of the endothelial cells. Endothelial cells from the venule begin degrading the surrounding basement membrane and extracellular matrix (ECM), endothelial cells migrate towards the

angiogenic stimulus and form a capillary sprout (differentiation), lumen forms in the sprout followed by endothelial proliferation, capillary loops form by the joining up of sprouts, and
5 capillaries mature with the formation of a basement membrane and the inclusion of pericytes. These capillary cells, having 'sprouted' from the parent vessel, proliferate in response to growth factors and angiogenic agents in the surrounding to form
10 lumen and eventually new blood vessels.

There are several aspects to angiogenesis, including cell elaborating factors: endothelial, tumor, macrophages, and mast cells, which serve as angiogenic stimuli, the responding cell(s) and the
15 supporting ECM components. However, the ECM is thought to play a key role in the physiology of angiogenesis. The ECM is made up of three key components: the insoluble components, like collagen and elastin, form the basic structure or
20 the architecture of the matrix and provide the critical tensile strength to the tissues; the soluble components, such as heparin, heparin sulfate, and dermatan sulfate, hydrate the matrix; and the transient components, like growth factors,
25 cytokines, and adhesion molecules, which are frequently solubilized by the soluble components, act in a localized fashion to modulate the physiology of the tissue.

Of the different ECM components, the
30 soluble components or the proteoglycans (heparin sulfate proteoglycan or HSPG) play a critical role in mediating the function of the transient components. They serve as obligatory mediators in the signaling mechanism of many angiogenic factors,
35 in the interactions between cell surface and the protein components in the matrix, and also between the cytoskeleton and the matrix proteins

(Rapraeger, (1993), In *Molecular and Cellular Aspects of Basement Membranes*, edited by Rohrbach (San Diego, CA, Academic Press), 267). They have been proven to play key roles in presentation of
5 heparin binding growth factors, for example, bFGF, aFGF, and VEGF, to their receptors on various cells including endothelial cells, fibroblasts and smooth muscle cells (Rapraeger, et al., (1991) Science, 252, 1708; Yayon, et al., (1991) Cell 64, 841;
10 Ornitz, et al., (1992) Mol. Cell Biol., 12, 240; Olwin and Rapraeger, (1992) J. Cell Biol. 118, 631; Gitay-Goren, et al., (1992) J. Biol. Chem. 267, 6093), thereby influencing a variety of cellular processes ranging from cell adhesion, migration,
15 growth, proliferation, differentiation, etc. (Rapraeger, (1993) Current Opinion in Cell Biol. 5, 844; Jalkanen, et al., (1993) Trends Glycosci. Glycotechnol. 5, 107; Rapraeger, (1993); Wight, et al., (1992) Curr. Opin. Cell Biol. 4, 793).

20 Heparin and heparan sulfate represent a class of glycosaminoglycans characterized by a linear polysaccharide of D-glucosamine (1→4) linked to hexuronic acid (Linhardt, R.J. (1991) Chem. Ind. 2, 45-50; Casu, B. (1985) Adv. Carbohydr. Chem.
25 Biochem. 43, 51-134). Heparin and heparan sulfate are complex carbohydrates that play an important functional role in the extracellular matrix of mammals. These polysaccharides modulate and regulate tissue level events that take place either
30 during development under normal situations or wound healing and tumor metastasis under pathological conditions.

Much of the current understanding of heparin and heparan sulfate sequence has relied on
35 studies of their biosynthesis (Linhardt, R.J., Wang, H.M., Loganathan, D., and Bae, J.H. (1992) Biol. Chem. 267, 2380-2387; Lindahl, U., Feingold,

D., and Roden, L. (1986) Trends Biochem. Sci. 11, 221-225; Jacobson, I., and Lindahl U. (1980) J. Biol. Chem. 255, 5094-5100; Lindahl, U., and Kjellen, L. (1987) in *The Biology of Extracellular Matrix Proteoglycans* (Wight, T.N., and Mecham R., eds) pp. 59-104, Academic Press, New York).

Heparan sulfate, which is chemically almost indistinguishable from heparin, is believed to be present on virtually all cell surfaces. Heparin-like molecules are associated with membrane proteins and are called proteoglycans. Proteoglycans are predominantly found in the extracellular matrix (ECM) and function in cell adhesion to the extracellular matrix. It is increasingly recognized that heparin is more than a mere structural oligosaccharide as it interacts with other key proteins of the extracellular matrix, such as laminin, fibronectin and collagen, and helps to define the physiological properties of the matrix. There has been speculation that heparin plays some role in angiogenesis, primarily due to heparin's role as an inhibitor of angiogenesis, in conjunction with cortisone. Also, heparin interacts with an array of cytokine-like growth factors present in the extracellular matrix, by facilitating their biochemical interaction with receptors and by protecting them from proteolytic degradation.

The growth factors in the matrix play a critical role in the angiogenic stimulus. For example, bFGF is chemotactic for endothelial cells, induces secretion of plasminogen activator and collagenase (Sato et al., (1988) J. Cell Biol. 107, 1119), initiates capillary tube formation *in vitro*, and is a potent stimulator of angiogenesis in *in vivo* models like the CAM assay, the corneal pocket assay, and tumor models in animals (Zetter, (1988)

93, 159S). Central to this signaling pathway is the tri-molecular interaction of the growth factor, the cell surface receptor and the polysaccharide heparin sulfate (Klagsbrun, M. & Baird, (1991). Cell 5 67, 229). Further, the multiplicity of cellular responses to growth factor stimuli, in the form of changes in cell shape, cell adhesion, migration, proliferation and differentiation into formed capillaries are believed to involve interactions of 10 growth factors with the ECM, particularly the participation of the HSPGs. Heparin potentiates the biological activity of aFGF, as reported by Thornton, et al., Science 222, 623-625 (1983), possibly by potentiating the affinity of aFGF for 15 its cell surface receptors, as reported by Schreiber, et al., Proc. Natl. Acad. Sci. USA 82, 6138-6142 (1985). Heparin protects aFGF and bFGF from degradation by heat, acid and proteases, as reported by Gospodarowicz and Cheng, J. Cell 20 Physiol. 128, 475-484 (1986); Rosengart, et al., Biochem. Biophys. Res. Commun. 152, 432-440 (1988); and Lobb Biochem. 27, 2572-2578 (1988). bFGF is stored in the extracellular matrix and can be mobilized in a biologically active form by heparin 25 or heparan sulfate, as reported by Vlodavsky, et al., Proc. Natl. Acad. Sci. USA 84, 2292-2296 (1987) and Folkman, et al., Am. J. Pathol. 130, 393-400 (1988). The binding of FGF to heparan sulfate is a prerequisite for the binding of FGF to 30 its high affinity receptor on the cell surface, as reported by Yayon, et al., Cell 64, 841-848 (1991) and Papraeger, et al., Science 252, 1705-1708 (1991). A specific heparan sulfate proteoglycan has been found to mediate the binding of bFGF to 35 the cell surface, as described by Kiefer, et al., Proc. Natl. Acad. Sci. USA 87, 6985-6989 (1990).

Although a number of these studies have focused on the role of heparin-like molecules in neovascularization, little is known about the role of heparin-degrading enzymes in neovascularization.

5 Heparin-like molecules such as heparin and heparan sulfate bind several cytokines, which are angiogenic, and modulate their function either by stabilizing them or by controlling their bioavailability, as reported by Folkman and Shing, J. Biol. Chem. 267, 10931-10934 (1992). These

10 molecules have been shown by Klagsbrun and Baird, Cell 67, 229-231 (1991), to act as low affinity receptors on cell surfaces and to facilitate growth factor activity and receptor binding. Heparin

15 lyases are a general class of enzymes that are capable of specifically cleaving the major glycosidic linkages in heparin and heparan sulfate. Three heparin lyases have been identified in *Flavobacterium heparinum*, a heparin-utilizing

20 organism that also produces exoglycouronidases, sulfoesterases, and sulfamidases that further act on the lyase-generated oligosaccharide products (Yang, et al. J. Biol. Chem. 260, 1849-1857 (1987); Galliher, et al. Eur. J. Appl. Microbiol. Biotechnol. 15, 252-257 (1982). These lyases are

25 designated as heparin lyase I (heparinase, EC 4.2.2.7), heparin lyase II (heparinase II, no EC number) and heparin lyase III (heparitinase EC 4.2.2.8). The three purified heparin lyases differ

30 in their capacity to cleave heparin and heparan sulfate: Heparin lyase I primarily cleaves heparin, heparin lyase III specifically cleaves heparan sulfate and heparin lyase II acts equally on both heparin and heparan. Several *Bacteroides* sp.

35 (Saylers, et al. Appl. Environ. Microbiol. 33, 319-322 (1977); Nakamura, et al. J. Clin. Microbiol. 26, 1070-1071 (1988)) also produce heparinases. A

heparinase has also been purified to apparent homogeneity from an unidentified soil bacterium by Bohmer, et al. J. Biol. Chem. 265, 13609-13617 (1990). The *in vivo* effect of these heparinases, other than on the degradation of heparin, has never been determined.

Inhibition of angiogenesis can occur at any of the above key junctures. A chemical agent which prevents the continued spread of vascularization could have broad applicability as a therapy for those disease in which neovascularization plays a prominent role.

It is an object of the present invention to provide pharmaceutical compositions, and method of use thereof, based on heparinases, for the treatment of diseases involving abnormal angiogenesis.

It is a further object of the present invention to provide pharmaceutical compositions, and method of use thereof, based on heparinases, for inhibition of capillary endothelial cell proliferation and migration.

It is another object of the present invention to provide topical and controlled release pharmaceutical compositions, and methods of use thereof, based on heparinases, for inhibition of angiogenesis.

Summary of the Invention

Pharmaceutical compositions for delivering an effective dose of a heparinase inhibiting angiogenesis to a desired site are described. These compositions are based on the discovery that heparinase alone can inhibit angiogenesis. The heparin-degrading enzymes heparinases I and III have been demonstrated to inhibit both neovascularization *in vivo* and proliferation of

capillary endothelial cells mediated by basic fibroblast growth factor *in vitro*, and cause dramatic loss in binding of basic fibroblast growth factor to these cells. Heparinase II did not
5 inhibit neovascularization *in vivo*, had marginal effect on endothelial cell proliferation and the binding of basic fibroblast growth factor to these cells, but is very useful in the alteration of smooth muscle cell proliferation.

10 The effective dosage for inhibition of angiogenesis *in vivo*, defined as inhibition of capillary endothelial cell proliferation and migration and blood vessel ingrowth, can be extrapolated from *in vitro* inhibition assays. In
15 *vitro* assays have been developed to screen for inhibition of angiogenesis. Events that are tested include proteolytic degradation of extracellular matrix and/or basement membrane; proliferation of endothelial cells, migration of endothelial cells,
20 and capillary tube formation. Effective dosages range from 10 nM to 100 nM heparinase I or III for inhibition of capillary endothelial cell proliferation and between 1 μ g and 4 μ g heparinase I or III for inhibition of localized angiogenesis.
25 The effective dosage is dependent not only on the heparinase, but also on the method and means of delivery, which can be localized or systemic. For example, in some applications, as in the treatment of psoriasis or diabetic retinopathy, the inhibitor
30 is delivered in a topical ophthalmic carrier. In other applications, as in the treatment of solid tumors, the inhibitor is delivered by means of a biodegradable, polymeric implant.

Brief Description of the Drawings

Figures 1a, 1b, 1c and 1d are micrographs showing inhibition of angiogenesis by heparinase in the chick chorionic membrane assay. Figure 1a is the CAM with heparinase I containing disk: 100% of the eggs tested (n=12) of several different batches of heparinase I, had avascular zones; Figure 1b is a normal CAM containing an empty methylcellulose disk; Figure 1c are histological sections of day 8 normal CAMs x800; and Figure 1d heparinase I treated CAM.

Figure 2 is a graph of capillary endothelial cells growth (10^3) versus heparinase concentration ($\mu\text{g/ml}$), in the presence (open triangles) or absence (X) of FGF.

Figure 3a and 3b are graphs of the percent control of HSPG (a) or bFGF (b) binding to bovine capillary endothelial (BCE) cells versus heparinase (nM).

Detailed Description of the Invention

It has been discovered that heparinases directly inhibit neovascularization. It has also been observed that heparinases of different substrate specificity differentially affect BCE proliferation and bFGF binding to these cells. For example, the substrate specificities of heparinases I, II and III from *F. heparinum* may control either the availability or the removal of unique heparin fragments essential for neovascularization. Heparinase III acts at the more "heparan sulfate-like regions" of the endothelial cell polysaccharide, leaving behind intact bFGF binding sites, and heparinase I cleaves the "heparin-like regions" of the polysaccharide containing the bFGF binding sites; thus both enzymes differentially modulate the availability of specific bFGF binding

sites required for bFGF-mediated BCE proliferation, as described by Nader, et al. Proc. Natl. Acad. Sci. U.S.A. 84, 3565-3569 (1987); Lindblom, et al. Biochem J. 279, 821-829 (1991); and Turnbull, et al. J. Biol. Chem. 267, 10337-10341 (1992).
However, since heparinase II non-specifically cleaves the polysaccharide primarily into disaccharides, this enzyme probably does not hit enough sites to alter heparan sulfate structure significantly enough to affect bFGF binding and activity.

In complete contrast to the observations described herein, it has been suggested by Folkman, J. & Shing, Y. J. Biol. Chem. 267, 10931-10934 (1992), that enzymes such as heparinases, which degrade ECM structural components, have a role in aiding the release of growth factors sequestered in the matrix and facilitating EC proliferation and neovascularization in vivo. For example, it has been proposed by Vlodavsky, et al. Cancer Met. Rev. 9, 203-226 (1990), that heparinases degrade the basement membrane serving as a reservoir for bFGF to initiate tumor or wound neovascularization.

As described herein, however, these enzymes appear to have another role wherein they inhibit neovascularization by depleting the low affinity receptors that are critical for bFGF mediated proliferation of endothelial cells and hence neovascularization. In addition to bFGF, several endothelial cell cytokines have been found by Gitay-Goren, et al. J. Biol. Chem. 267, 6093-6098 (1992) and Folkman, J. & Klagsbrun, M. Science 235, 442-447 (1987), to bind and be modulated by heparin-like molecules; thus heparinases could act on wide spectrum of heparin-dependent angiogenic factors. The mechanism described here for specific heparinase-mediated inhibition of bFGF binding and

activity on BCEs is believed to be reflective of a general system for the control of capillary endothelial cell proliferation and neovascularization and therefore subject to regulation using any molecule having heparinase-like activity.

I. Heparinases (heparin lyases).

Sources of Heparinase

A variety of heparinases are produced in bacteria, especially *Flavobacterium heparinum*, which produces three distinct heparinases, heparinase I, heparinase II, and heparinase III. As described herein, heparinase I and heparinase III are potent inhibitors of neovascularization. In the preferred embodiment, only heparinase purified of other activities or contaminants is used. Although heparinase is commercially available, it is preferred to use a more highly purified form, especially when derived from *F. heparinum*, since the bacteria produces a number of enzymes other than heparinase, such as chondroitinase, as well as the three very different forms of heparinase. Reports of studies conducted with the commercially available forms of heparinase have contained conflicting results and non-reproducible data, presumably due to contamination with other materials.

As used herein, "heparinase" includes the enzymes derived from natural sources, as well as those which have been modified naturally or synthetically to alter their binding affinity and/or catalytic activity.

Purification, composition and specificity of Heparinase I, II, AND III from *Flavobacterium heparinum*.

Heparin lyase I (heparinase, EC 4.2.2.7), heparin lyase II (no EC number), and heparin lyase

III (heparitinase, EC 4.2.2.8) have molecular weights (by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and isoelectric points (by isoelectric focusing) of M_r 42,800, pI 9.1-9.2, M_r 70,800, pI 9.9-10.1, respectively.

A method for purification is described below.

Materials

Enzyme assays and absorbance measurements were done on a UV 160 spectrophotometer from Shimadzu connected to a Fisher Scientific Isotamp model 9100 refrigerated circulating water bath. Fermentations were performed in a two-liter stirred tank fermenter from Applikon. Centrifugation was done on a Sorvall RC-5 refrigerated centrifuge in a GSA rotor from Du Pont. HPLC was performed using a LDC Milton-Roy Constametric IIIG pump, a Rheodyne 7125 injector, a Jule Linear Gradient Former, and an ISCO model UA-5 absorbance monitor with a 280-nm filter. The hydroxylapatite HPLC column 1x30 cm connected in series with a 1x5 cm guard column was from Regis, the Mono-S FPLC column was from Pharmacia LKB Biotechnology Inc., the C_{18} column was from Vydac, and the Bio-Sil gel permeation HPLC column was from Bio-Rad. The capillary zone electrophoresis system and the silica capillaries were from Dionex. The Mini-Protein II electrophoresis chamber, a model 1405 horizontal electrophoresis cell, and a model 1420B power source were from Bio-Rad. The tube gel electrophoresis equipment was from E-C Apparatus Corp. The precast agarose IEF gels were from Isolabs, and the prestained molecular weight markers and the Rapid CoomassieTM stain were from Diversified Biotech. The Bio-Gel HT hydroxylapatite was from Bio-Rad and the QAE-Sephadex was from Sigma. Pressure filtration units

and 25- and 43-mm PM-10 filters were from Amicon. Heparin (porcine mucosal sodium salt) was from Celsus, heparan sulfate, dermatan sulfate, and chondroitin sulfate A, C, D, and E were from
5 Seikagaku. Bovine serum albumin, lactose, protamine (free base), bromphenol blue, naphthol red, cytochrome c (bovine heart type VA), hyaluronic acid, CAPS, bis-Tris, HEPES, TES, dithiothreitol, MOPS, mercaptoethanol,
10 iodoacetamide, and trypsin were for Sigma. The Coomassie reagent for the protein assay was from Bio-Rad. All water used in reagents was deionized and distilled in glass.

Assays

15 The spectrophotometer was adjusted to the optimum temperature of the particular lyase being assayed. A 700 μ l quartz microcuvette containing 400 μ g of substrate in 50 mM sodium phosphate buffer (containing 100 mM sodium chloride for
20 heparin lyase I) was thermally equilibrated. A measured quantity of lyase was added, bringing the final volume to 400 μ l and the cuvette was gently mixed. The microcuvette was then immediately returned to the spectrophotometer and the change of
25 absorbance at 232 nm was measured at 10 seconds intervals over 3 min. The activity was measured from the change of absorbance/unit time using an extinction coefficient of 3800 M⁻¹ for products. The specific activity was then calculated by
30 dividing the micromoles of product produced per minute by the milligrams of protein in the cuvette. The molecular weights used for heparin, heparan sulfate, and the chondroitin sulfates were 14,000, 20,000 and 25,000, respectively. Protein
35 concentration was measured by the Bradford assay based on a bovine serum albumin standard curve.

Fermentation and Enzyme Recovery

F. heparinum (Payza, A.N., and Korn, E.D. (1956) Nature 177, 88-89) (ATCC 13, 125) was stored at -70°C in a defined medium containing dimethyl sulfoxide (Me₂SO) (Zimmermann, J.J., Oddie, K., Langer, R., and Cooney, C.L. (1991) Appl. Biochem. Biotech. 30, 137-148). The organism was grown in a two liter stirred tank fermenter on heparin as the sole carbon source in defined medium by the method of Galliher, P.M., Cooney, C.L., Langer, R.S., and Linhardt, R.J. (1981) Appl. Environ. Microbiol. 41, 360-365). From 5 liters of fermentation broth, an 80 g wet cell pellet was obtained by centrifugation for 15 min at 12,000 x g at 4°C. This pellet was suspended in 500 ml of 10 mM sodium phosphate buffer at pH 7.0 and 4°C. Cell suspension (20 ml at a time) was placed into a 50-ml stainless steel cup and sonicated with cooling for 10 min at 100 watts using a 40% pulsed mode. The disrupted cells were centrifuged at 12,500 x g for 30 min at 4°C and the pellet discarded. The 500 ml of supernatant, obtained by sonication and centrifugation, contained 16.3 mg/ml protein. Protamine free base (2.0 g) was dissolved in 20 ml of 10 mM sodium phosphate buffer, pH 7.0, and added dropwise with stirring to the 500 ml of supernatant. Centrifugation at 10,000 x g, at 4°C for 20 min, removed the precipitated DNA and gave 510 ml of supernatant.

Electrophoresis

Materials

Electrophoresis was performed using a Mini-Protean II electrophoresis cell from Bio-Rad (Richmond, Ca., U.S.A.). Acrylamide and N,N'-methylene bisacrylamide were from International Biotechnologies Inc. (New Haven, CT, U.S.A.) or used as a prepared 40% acrylamide

solution that is 37.5 acrylamide:1 N,N'-methylene bisacrylamide (Fischer Scientific, Fairlawn, NJ, U.S.A.). Tris {hydroxymethyl} aminomethane (Tris) was from Bio-Rad (Richmond, CA, U.S.A.). N,N,N',N'-
5 Tetramethylethylenediamine (TEMED) was from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Ammonium persulfate (APS) and glacial acetic acid were from Mallinckrodt Inc. (Pads, KY, U.S.A.). Urea and glycerol were from Fisher
10 Scientific (Fair Lawn, NJ, U.S.A.). Sodium dodecyl sulfate (SDS) was from BDH Chemicals, Ltd. (Poole, England). Naphthol red was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 2- β -mercaptoethanol was from EM Science (Gibbstown, NJ, U.S.A.).
15 Bromophenol blue was from MCB Manufacturing Chemists, Inc. (Cincinnati, OH, U.S.A.). Molecular Weight Standards and Rapid Coomassie Stain were from Diversified Biotech (Newton Centre, MA, U.S.A.)

20 SDS-polyacrylamide gel electrophoresis
(PAGE)

Separating gels (12% acrylamide, 10% SDS) were prepared by mixing 4.35 mL distilled water, 2.5 mL of 1.5 M Tris, pH 8.8 and 3.0 mL of a
25 commercially prepared solution of 37.5 acrylamide:1 N,N'-methylene bisacrylamide (Fischer Scientific, Fairlawn, NJ, U.S.A.) as described above. This solution was degassed under vacuum for at least 15 minutes. Next, 50 μ L of APS (10%) and 5 μ L of
30 TEMED were added to the monomer solution to initiate polymerization. The gel solution was quickly poured between two glass plates separated by 0.75 mm spacers, overlaid with distilled water saturated gamma-butanol and allowed to polymerize
35 at 25°C for 60 minutes.

Stacking gel was prepared by mixing 6.4 mL distilled water, 2.5 mL 0.5 M Tris, pH 6.8, 1.0 mL acrylamide/Bis solution (Fischer Scientific), 50 μ L

APS (10%) and 10 μ L TEMED. The gamma-butanol was removed from the separating gel, the gel was rinsed with distilled water and the stacking gel solution was carefully added to the top of the separating gel. A well-forming electrophoresis comb was inserted in the stacking gel prior to polymerization. The stacking gel was allowed to polymerize for 60 minutes and the well-forming comb was removed just prior to loading of the samples.

Sample buffer was prepared by mixing 4.0 mL distilled water, 1.0 mL 0.5 M Tris, pH 6.8, 0.8 mL glycerol, 1.6 mL SDS (10%), 0.4 mL 2- β -mercaptoethanol and 0.2 mL bromophenol blue (0.05% W/V). Samples and molecular weight standard markers for electrophoresis were diluted 1:4 in sample buffer and heated for 4 minutes at 100°C just prior to loading into the wells formed earlier in the stacking gel. Running buffer (0.125 M Tris, 1.0 M glycine, 0.5% SDS, pH 8.3) was carefully overlaid on the stacking gel and the electrophoresis was conducted at a constant voltage of 200 V until the bromophenol blue marker moved to within 0.3 cm of the bottom of the gel (typically about 45 minutes). Following electrophoresis, the gels were either electro-transferred to nitrocellulose membranes or were stained with Rapid Coomassie Stain for 45 minutes followed by destaining with a 7.5% methanol/5% acetic acid solution.

Purification of heparin Lyases from F. heparinum

Batch Hydroxylapatite Adsorption and Release

The 510 ml of supernatant containing 15.6 mg/ml protein, used directly without freezing, was divided equally into four 250 ml polypropylene centrifuge containers and placed in an ice bath. Dry hydroxylapatite (HA) (20 g) was added to each

container, gently stirred, lightly compacted by centrifugation at 1000 x g for 2 min at 4°C, and the supernatant was decanted away from the HA matrix. The HA-bound protein was then resuspended in buffers having increasing concentrations of sodium phosphate and sodium chloride and recompactd by centrifugation. The supernatants were again decanted away from the matrix and assayed for enzyme activity and protein concentration. The buffers used to wash the HA matrix were prepared by mixing a solution of 10 mM sodium phosphate buffer at pH 6.8, with a solution of 250 mM sodium phosphate buffer at pH 6.8, containing 500 mM sodium chloride in ratios of 6:0, 5:1, 4:2, 3:3, 2:4, and 0:6 (v/v) at 4°C. The protein supernatant solutions were placed in dialysis tubing having a molecular weight cut-off of 14,000 and dialyzed overnight at 4°C against 50 mM sodium phosphate buffer at pH 7.0.

QAE-Sephadex Chromatography

Lyase activity purified by batch HA was used immediately without freezing. A quaternary ammonium ethyl (QAE)-Sephadex chromatography step was performed at 4°C. Three batch HA-purified fractions (4:2; 3:3, and 2:4), having a total volume of 1.5 liters, containing more than 89% of the activity toward heparin and 88% of the activity toward heparan sulfate were consolidated (1.81 mg/ml protein and 1.72 units/ml toward heparin and 2.16 units/ml toward heparan sulfate) and applied directly in equal portions to three columns (2.5 x 20 cm) containing 600 ml of QAE-Sephadex. The QAE-Sephadex columns had been previously equilibrated with 50 mM sodium phosphate buffer, pH 7.0, at 4°C. Each column was then washed with 1-column volume of 50 mM phosphate buffer, pH 7.0, at 4°C. The fractions containing lyase activity that passed

through the columns without interaction were collected and combined. The 2.6 liters of eluent was then concentrated to 63 ml (containing 8.23 mg/ml of protein) by Amicon pressure filtration at 60 psi and 4°C using a 43 mm PM-10 membrane (10,000 molecular weight cut-off).

Hydroxylapatite HPLC

The 63 ml of QAE-Sephadex-purified and concentrated solution was divided into twelve 5 ml aliquots and stored at -70°C until needed. A 5 ml sample (43 mg of protein) was removed from the freezer, allowed to thaw at room temperature, and, using a 5 ml loop, injected onto a HA HPLC column. The HA-HPLC column had been equilibrated with 50 mM sodium phosphate buffer, pH 7.0. After loading the sample, the column was washed with 50 mM sodium phosphate buffer, pH 7.0, at 0.5 ml/min, for 20 min. A 60 ml linear gradient, from 50 mM sodium phosphate, pH 7.0, to 50 mM sodium phosphate buffer containing 750 mM sodium chloride, pH 7.0, was used to elute the column. The elution was monitored continuously at 280 nm. After the gradient was complete, the column was washed with 5.0 ml of 50 mM sodium phosphate containing 1 M sodium chloride, pH 7.0, to remove tightly bound proteins, and then re-equilibrated with the 50 mM sodium phosphate buffer, pH 7.0. This fractionation step was repeated with the 11 remaining aliquots. The fractions corresponding to heparin lyase I, heparin lyase II, and heparin lyase III from each of the 12 fractionations were pooled, dialyzed against 20 volumes of 50 mM sodium phosphate buffer, pH 7.0, for 12 h at 4°C, and concentrated at 60 psi and 4°C using Amicon pressure filtration equipped with PM-10 membranes. The three lyase preparations were each divided into 1-ml aliquots and frozen at -70°C.

Mono-S FPLC of heparin Lyases I and III

The concentrated heparin lyase I and heparin lyase III preparations, isolated from HA-HPLC, were taken from the -70°C freezer, thawed at room temperature, and applied to a Mono-S FPLC HR 5/5 cation-exchange column equilibrated with 50 mM sodium phosphate buffer, pH 7.0. A portion of each lyase preparation, 350 µl containing 1.75 mg of protein, was injected and the column washed at 1 ml/min for 5 min with 50 mM sodium phosphate buffer, pH 7.0, to elute non-interacting proteins. A linear gradient from 50 mM sodium phosphate buffer, pH 7.0, to 50 mM sodium phosphate containing 500 mM sodium chloride, pH 7.0, was used and the elution was monitored at 280 nm. The active heparin lyase I and heparin lyase III fractions were dialyzed at 4°C against 200 mM sodium phosphate buffer, pH 7.0, for 12 h and concentrated using Amicon Pressure Filtration with a PM-10 membrane (molecular weight cut-off 10,000).

Gel Permeation HPLC

The heparin lyase I and III preparation obtained from Mono-S FPLC and the heparin lyase II preparation obtained from HA-HPLC were applied to a Bio-Sil gel permeation chromatography (GPC) HPLC column (1 x 25 cm) that had been equilibrated with 200 mM sodium phosphate buffer, pH 7.0. Each lyase was injected (250 µl samples containing 800 µg of protein for heparin lyases I and III; 200 µl samples containing 1.5 mg of protein for heparin lyase II), eluted at a flow rate of 1 ml/min and absorbance at 280 nm was measured. This separation was repeated five times for heparin lyases I, II, and III. The active fractions were pooled together and assayed for lyase activity and protein concentration. Each heparin lyase was dialyzed against 50 mM sodium phosphate buffer, pH 7.0,

concentrated at 60 psi and 4°C using pressure filtration with 25 mm PM-10 membranes (molecular weight cut-off 10,000), and subdivided into 10 µl aliquots and stored at -70°C.

5 Characterization of the Three heparin
Lyases

Assessment of Purity by Electrophoresis

 Discontinuous SDS-PAGE was performed on the
three heparin lyases using a modification of a
10 procedure previously described by Laemmli. The
gels were fixed with 12% (w/v) trichloroacetic
acid, rinsed with deionized, distilled water and
stained with a Rapid Coomassie Stain solution, and
destained.

15 IEF gel electrophoresis was run on pre-cast
agarose gels (85 x 100 mm). Two electrode wicks
were wetted with 1 M phosphoric acid (anolyte) and
1 M sodium hydroxide (catholyte). Electrophoresis
was at 5 watts for 5 min, then at 10 watts for 1 h
20 until the voltage was constant at 1200 V. The gel
was immediately fixed in 15% aqueous
trichloroacetic acid, blotted and rinsed with
water, dried overnight, stained by using Coomassie
G-250, and destained.

25 Continuous acid-urea gel electrophoresis
was performed in 10% polyacrylamide tube gels.
Heparin lyase I-III samples (10 µg) were prepared
in acetic acid-urea buffer containing glycerol and
naphthol red as a tracking dye. Electrophoresis
30 was at a constant current of 2.5 mA/tube gel. The
proteins were run toward the cathode for
approximately 2 h, until the 100 µg of cytochrome c
standard (a brown band) was at the bottom of its
tube. Staining and destaining were accomplished as
35 described for SDS-PAGE.

 Capillary zone electrophoresis on the three
heparin lyases used a Dionex Capillary
Electrophoresis System on a 375 µm x 70-cm

capillary by a previously published method for protein analysis (23) in 20 mM CAPS containing 10 mM potassium chloride, pH 11.0, at 20 kV at room temperature and detection was by absorbance at 280 nm. Heparin lyase I-III samples (20 nl), each containing 2.74, 2.07, and 2.45 mg/ml, respectively, were analyzed.

Mass spectrometry

Heparinases was purified as described by Sasisekharan, et al. Proc. Natl. Acad. Sci. U.S.A. 90, 3660-3664 (1993), the teachings of which are incorporated herein and as summarized above. They were extensively desalted using a Centricon P-30 (molecular weight cut-off of 30 kDa) (Amicon, MA) and the purity of the peak was determined by high pressure liquid chromatography using a Vydac C₁₈ reverse-phase column in a HP 1090 (Hewlett Packard, CA), with diode array detection, in a gradient of 0 to 80% acetonitrile in 0.1% TFA for 60 min. Protein was monitored at 210 and 277 nm. Heparinase I, II and III were collected in a microfuge tube and lyophilized (VirTis Freeze Mobil Model 12, VirTis Inc., NY). Protein concentration was determined by use of the Micro BCA reagent (Pierce Inc., IL) relative to a bovine serum albumin standard.

Mass spectrometry was performed on the heparinase preparations to determine purity, homogeneity and molecular mass. About 2 µg of heparinase were mixed with 1 µl of 10 mg/ml sinapinic acid (in 80% acetonitrile 0.1% TFA in water), in equal vol./vol. ratio and then analyzed using laser desorption mass spectrometry (Laser MAT, Finnigan, CA). The results demonstrate the purity, homogeneity, and expected molecular mass.

II. Assays for determining effectiveness and effective concentration of angiogenesis inhibitors *in vivo*.

Assays were used to test heparinase for endothelial cell proliferation, FGF binding to endothelial cell receptors, and inhibition of angiogenesis *in vivo*.

The essential elements of these assays include using endothelial cells as the assay target and stimulation of the endothelial cells with known angiogenesis factors to compare a putative inhibitor's effect in unstimulated, as compared to stimulated, endothelial cells. Endothelial cells that can be used include capillary endothelial cells and umbilical vein endothelial cells. Factors which can be used to stimulate the endothelial cells include acidic or basic fibroblast growth factor.

The following assays are based on assays described in the prior art that have been modified for the purpose of screening heparinase for inhibition of angiogenesis *in vivo*.

Example 1: Effect of heparinase in the Chick chorioallantoic membrane bioassay.

The chick chorioallantoic membrane assay (CAM), described by Taylor and Folkman, Nature (London) 297, 307-312 (1982), is used to determine whether the compound is capable of inhibiting neovascularization *in vivo*. The effect of the compound on growing embryonic vessels is studied using chick embryos in which capillaries appear in the yolk sac at 48 h and grow rapidly over the next 6-8 days.

On day 3 of development, fertilized chick embryos are removed from their shells and placed in plastic petri dishes (1005, Falcon). They are maintained in humidified 5% CO₂ at 37°C. On day 6, 4 µg samples of purified heparinases are mixed in

methycellulose disks and applied to the surfaces of the growing CAMs above the dense subectodermal plexus. Following a 48 hour exposure of the CAMs to heparinases, avascular zones, free of
5 capillaries and small vessels are observed using a binocular dissecting microscope at $\times 7^{10}$ magnification.

Tissue specimens are fixed in formalin at room temperature and rinsed in 0.1 M cacodylate
10 buffer pH 7.4. The specimens are embedded in JB-4 plastic (Polysciences) at 4°C and 3 μ m sections are cut using a Reichert 2050 microtome. Sections are stained with toluidine blue and micrographs taken on a Zeiss photomicroscope using Kodak TM x100 and
15 a green filter.

The results are shown in Figures 1a, 1b, 1c, and 1d for heparinase I. On day 6, heparinase I (100 pmoles) was mixed in methycellulose disks and applied to the surfaces of the growing CAMs
20 above the dense sub-ectodermal plexus. Figure 1a is a photograph of the CAM with heparinase I containing disk injected with India ink: 100% of the eggs tested (n=12) of several different batches of heparinase I had avascular zones; Figure 1b is a
25 normal control CAM containing an empty methycellulose disk; Figure 1c is a histological section of a day 8 normal CAM $\times 800$; and Figure 1d is a histological section of a heparinase I treated CAM. The data for heparinase III was similar.

30 Purified samples of heparinases I and III (100 and 50 pmole respectively) in methycellulose discs caused 100% inhibition of embryonic neovascularization, resulting in large avascular zones, as shown in Figure 1a. In contrast,
35 heparinase II (50 pmole) and control CAMs implanted with empty methycellulose discs did not develop avascular zones, as shown by Figure 1b.

Histological analysis of heparinase I treated CAM revealed a mesoderm that was thinner than normal, containing dividing fibroblasts and nearly no capillary endothelial cells, as shown by Figures 1c and d.

Example 2: Assay for Inhibition of capillary endothelial cell proliferation.

Capillary EC proliferation in response to an angiogenic stimulus is a critical component of neovascularization, as discussed by Ausprunk and Folkman, J. Microvasc. Res. 14, 153-65 (1977). By utilizing the specific cells involved in angiogenesis, and stimulating them with known angiogenesis factors, in this case basic fibroblast growth factor (bFGF), as reported by D'Amore and Klagsbrun, J. Cell Biol. 99, 1545-1549 (1984), it is possible to mimic the angiogenesis process in vitro. This type of assay is the assay of choice to demonstrate the stimulation of capillary EC proliferation by various angiogenic factors and inhibitors, as reviewed by Shing, et al., Science 223, 1296-1298 (1984).

Capillary EC were stimulated to proliferate by basic fibroblast growth factor (bFGF), a known angiogenic molecule, and then challenged with varying concentrations of heparinase. Following a three day incubation period, the number of endothelial cells was measured by electronic cell counting. Two concentrations of bFGF were used in the assay and a control was performed, where the effect of heparinase in the absence of bFGF was also investigated.

Capillary ECs were plated in DME/5 onto gelatin-coated (1.5% gelatin, wt/vol [Difco, Detroit, MI] 24-well tissue culture dishes (nunc, Roskilde, Denmark). At the indicated times, inhibitor samples and acidic FGF (aFGF) (F.G.F. Co., La Jolla, CA) were added to some of the wells.

Wells containing PBS alone and PBS and aFGF were included as controls. These substances were added simultaneously in volumes that did not exceed 10% of the final volume. After incubation with the test substances for the designated times, medium was aspirated, cells were washed with 0.5 ml of PBS, removed by trypsinization (0.5 ml 0.05% trypsin-0.02% EDTA; Gibco Laboratories), and counted electronically with a cell counter (Coulter Electronics, Hialeah, FL).

This cell counting assay was supported by an additional proliferation assay which measures the number of endothelial cells in culture on the basis of the colorimetric measurement of cellular acid phosphatase.

As seen from Figure 1 and Table 1, heparinase I is a potent inhibitor of bFGF-stimulated capillary EC proliferation. Capillary EC proliferation (upon stimulation by bFGF) measurement indicated that heparinase, at a 100 nM concentration, caused 100% inhibition of proliferation. On the other hand, at low heparinase concentration (300 pM), there was potentiation of bFGF mediated ECM proliferation by about 30%. Heparinase alone (in the absence of bFGF) has only an inhibitory effect on the EC growth and causes about 25% inhibition at a 100 pM concentration.

Heparinase III is also a potent inhibitor of bFGF-stimulated capillary EC proliferation. In fact, heparinase III seems more potent than heparinase I. At a concentration of approximately 70 nM, it is 100% inhibitory of the EC proliferation. Heparinase III also seems different than that of heparinase I, in action, due to the fact at low heparinase III concentrations (200 pM), there is a proliferation of EC of about 45%.

Heparinase II is marginally inhibitory. It does not have significant potentiating properties at concentrations similar to the concentrations at which heparinase I or III are inhibitory.

Table 1: Capillary Endothelial Cell Proliferation Assay demonstrating the Effect of heparinases.

Enzyme	0.005 μ g/ml	0.05 μ g/ml	0.5 μ g/ml	5.0 μ g/ml
Heparinase I	+12%	no effect	-25%	-100%
Heparinase II	-4%	-8%	no effect	-20%
Heparinase III	+12%	+45%	-60%	-95%

Example 3: Effect of heparinase on confluent BCE cells.

The results in examples 1 and 2 suggested that the effects of heparinases I and III on neovascularization *in vivo*, and BCE proliferation *in vitro*, reflect their ability to interfere with the action of bFGF. It is known that heparan sulfate (as a low affinity receptor) is essential for bFGF receptor (high affinity receptor) binding and activity, as reviewed by Klagsbrun, M. & Baird, A. Cell 67, 229-231 (1991); Baird, A. & Ling, N. Biochem. Biophys. Res. Commun. 142, 428-435 (1987); Moscatelli, D. J. Cell. Physiol. 131, 123-130 (1987); and Yayon, et al. Cell 64, 841-848 (1991), and the degradation of cell surface heparan sulfate with heparinase I results in inhibition of bFGF receptor binding, as reported by Baird, et al., Moscatelli, and Yayon, et al, and Nugent, M.A. & Edleman E. Biochemistry 31, 8876-8883 (1992). Furthermore, heparinase III treatment substantially reduced receptor binding, mitogenic activity, and blocked terminal differentiation of MM14 skeletal muscle cells, as reported by Rapraeger, et al. Science 252, 1705-

1708 (1991). These results suggest that the heparinases could alter the action of bFGF at the level of receptor binding. Therefore, the effects of heparinases I, II and III treatment on ^{125}I -bFGF receptor binding were investigated.

Methods and Materials.

BCEs were treated with the three heparinases for 30 min at 37°C, and then equilibrium binding of ^{125}I -bFGF to heparan sulfate and bFGF receptors was evaluated.

^{125}I -bFGF was prepared by a modification of the Bolton-Hunter procedure (Bashkin, et al., 1989; Kurokawa, et al., 1989). This technique has been demonstrated to produce active ^{125}I -bFGF as determined by its ability to bind heparin-Sepharose and to stimulate DNA synthesis in Balb/c3T3 and bovine capillary endothelial cells (Bashkin, et al., 1989; Kurokawa, et al., 1989). The specific activity of the ^{125}I -bFGF was assessed by stimulation of proliferation of Balb/c3T3 cells and was 25-100 $\mu\text{CV}/\mu\text{g}$.

Confluent capillary EC monolayers were prepared by plating 1×10^5 cells per 4 cm^2 well (12-well plates, Costar, Cambridge, MA) in DMEM, 10% calf serum (1 ml/well) and incubating at 37°C for 3-5 days. To initiate enzyme treatment, the medium was removed, the cells washed one time with DMEM (1 ml/well), and heparinase added at concentrations of 0, 0.1, 1, 10, or 100 nM in 0.25 ml DMEM containing 5 mg/ml BSA. Enzyme treatment was carried out at 37°C for 30 min. When treatment was completed the enzyme medium was removed and the monolayers washed twice (1 ml/well/wash) with cold (4°C) binding buffer (DMEM, 25 mM HEPES, 0.5% gelatin), and then incubated for 10 min at 4°C in 0.5 ml binding buffer to precool the cells. ^{125}I -bFGF (5 ng, 0.66 nM, $(1.25 \times 10^5 \text{ to } 5 \times 10^5 \text{ cpm})$) was

then added. The plates were incubated at 4°C for 2 h, at which point the binding buffer was removed and each well washed 3 times with cold binding buffer (1 ml/well/wash). The amount of ¹²⁵I-bFGF bound to HSPG and receptor was determined sequentially in each culture using a modification of the salt/acid washing technique of Moscatelli, D. J. Cell. Physiol. 131, 123-130 (1987) and Nugent, M.A. & Edleman E. Biochemistry 31, 8876-8883 (1992). ¹²⁵I-bFGF bound to HSPG was released by exposure to high salt buffer (2 M NaCl, 20 mM HEPES, pH 7.4; 0.5 ml/well for 5 s), and the ¹²⁵I-bFGF bound to receptors was extracted by incubation of the monolayers in low pH buffer (2 M NaCl, 20 mM sodium acetate, pH 4.0; 0.5 ml/well; 5 min) followed by a wash with the same buffer (0.5 ml/well). ¹²⁵I-bFGF was determined in all samples by counting in a 1272 Clinigamma gamma counter (LKB Nuclear, Gaithersburg, MD). The ¹²⁵I-bFGF bound that was not competed by an excess (5 µg; 55 nM) of unlabeled bFGF was defined as nonspecific and was subtracted from the experimental points. The number of cells attached to the culture plates before and after the salt and acid washes was similar.

Results.

¹²⁵I-bFGF binding to heparan sulfate proteoglycan (HSPG) and cell surface receptor on confluent capillary ECs treated with heparinases I, II and III in concentrations of 0.1, 1, 10, and 100 nM was performed. Binding of FGF to HSPG indicates lower affinity receptors; binding of FGF to the receptors indicates high affinity receptors. The results are shown in Figure 3a. The control binding level (100%) was 1592 ± 69 cpm for HSPG, and 821 ± 27 cpm for receptors. Similar results were observed in five separate experiments.

Heparinase treatment did not result in any change in cell viability, as determined by trypan blue exclusion.

It was found that heparinase treatment of capillary EC caused a significant loss in bFGF binding capacity in these cells (Figure 3a). Treatment with 125 nM heparinase I resulted in greater than 95% loss in HSPG binding sites on ECs accompanied by an over 80% decrease in receptor binding. The half maximal concentrations required to remove HSPG sites and reduce receptor binding were 0.7 nM and 1.5 nM respectively. The dose response for heparinase-induced inhibition of bFGF mitogenesis and bFGF receptor binding are similar, suggesting that these two activities may be related.

^{125}I -bFGF¹ (5 ng, 0.55 nM, $(1.25-5) \times 10^5$ cpm) was added directly to each well containing platelets. The platelets were incubated at 4°C for 2 hours, at which point the binding buffer was removed and each well washed 3 times with cold binding buffer (1 ml/well per wash). The amount of ^{125}I -bFGF bound to HSPG and CSR was determined sequentially in each culture using a modification of the salt/acid washing technique (Moscattelli, 1987; Nugent and Edelman, 1992). The ^{125}I -bFGF bound to HSPG was released by exposure to high salt buffer (2 M NaCl, 20 mM HEPES, pH 7.4; 0.5 ml/well for 5 seconds), and then the CSR bound ^{125}I -bFGF was extracted by incubation of the monolayers in low pH buffer (2 M NaCl, 20 mM Sodium Acetate, pH 4.0; 0.5 ml/well; 5 min) followed by a wash with the same buffer (0.5 ml/well). ^{125}I -bFGF was determined in all samples by counting in a 1272 Clinigamma gamma counter (LKB Nuclear, Gaithersburg, MD). The ^{125}I -bFGF bound that was not competed by an excess (5 μg ; 555 nM) of unlabeled bFGF was defined as

nonspecific and was subtracted from the experimental points. The number of cells attached to the culture plates before and after the salt and acid washes was not different.

5 Heparinase treatment of BCEs decreased bFGF binding to these cells in a dose-dependent fashion, as shown in Figure 3b. There was not only a significant loss of bFGF binding, but also a dramatic difference in the effects of the three
10 heparinases. The concentrations of heparinase I required to reduce bFGF binding to heparan sulfate and receptor sites by 50% were 0.5 nM and 1.5 nM, respectively. For heparinase II, IC_{50}
15 concentrations were 2 nM and 8 nM, respectively, for the heparan sulfate and the receptor binding sites. On the other hand, heparinase III was most potent in inhibiting the bFGF binding, with an IC_{50}
20 of 0.15 nM and 0.2 nM for the heparan sulfate and the receptor binding, respectively. Heparinase III potency is an order of magnitude greater than that of heparinase II; this could be due to slower kinetics of heparinase II compared to those of heparinase III. In the concentration range where
25 potentiation of BCE proliferation by heparinases III and I occurs, between 0.1 and 1 nM, bFGF binding to the cell surface heparan sulfate is eliminated by greater than 80% for heparinase III, and by approximately 60% for heparinase I; the
30 receptor binding is eliminated by only 50% for heparinase III and by approximately 20% for heparinase I. For the same concentration range of heparinase II, 0.1 to 1 nM, about 25% heparan sulfate binding sites and about 10% of the receptor binding are eliminated; heparinase II inhibition of
35 BCE proliferation is about 6%.

While heparinase II has a marginal effect on the BCE proliferation in the concentration

ranges of between 10 and 100 nM where heparinase
III and I dramatically inhibit BCE proliferation,
all three enzymes show similar effects in bFGF
binding to these cells. In the 10 to 100 nM
5 concentration range, almost all heparan sulfate
binding and about 90, 85 and 80% of the receptor
binding is eliminated for heparinases III, I and
II, respectively.

In summary, the effect of heparinase was
10 first tested on the proliferation of capillary
endothelial cells (ECs) *in vitro* in the presence
and absence of bFGF, a potent stimulator of EC
proliferation and angiogenesis. Heparinase I was a
powerful inhibitor of FGF-stimulated capillary EC
15 proliferation with an IC_{50} of 60 nM. At very low
concentrations of heparinase (14.2 pM and 142 pM)
this enzyme caused small but reproducible
potentiation of FGF stimulation.

To determine the cell specificity of
20 heparinase's anti-proliferative effect, it was
tested against bovine aortic endothelial cells,
bovine aortic smooth muscle cells (SMCs) and Balb/c
3T3 cells. Heparinase did not inhibit the
proliferation of any of these cell types even at
25 twice the IC_{50} concentration for capillary EC.
Heparinase II potentiated SMC proliferation in the
presence of platelet derived growth factor (PDGF);
heparinase I had no effect while heparinase III had
marginal effect in the potentiation of SMC
30 proliferation in the presence of PDGF. PDGF is the
mitogen of choice for stimulation of proliferation
of smooth muscle cells.

Basic fibroblast growth factor (bFGF) is a
potent mitogen for bovine capillary endothelial
35 cells (BCEs) and is considered an important
mediator of neovascularization. The three
heparinases were tested for their effect on BCE

proliferation *in vitro* in the presence and absence of bFGF. Heparinases I and III at IC_{50} of 21 nM and 6 nM, respectively, significantly inhibited bFGF-stimulated BCE proliferation, as shown in Figure 3b. It should be noted that, in the presence of bFGF, heparinases I and III potentiated BCE proliferation in very low concentration ranges (0.1 nM to 1 nM). While heparinase I potentiated up to about 20% and heparinase III potentiated up to approximately 40%, heparinase II did not have any potentiating effect, and its inhibition of the bFGF mediated proliferation was marginal (approximately 20% at 100 nM concentration). The anti-proliferative effect of heparinases I and III appears specific to BCE, in that these heparinases did not inhibit the proliferation of bovine aortic smooth muscle cells or NIH 3T3 cells (with bFGF as the mitogen), even when tested at twice the concentration of heparinase in the same assay.

Example 4: Safety of administration of heparinase for *in vivo* inhibition of retinal neovascularization in a rat model.

The role of heparinase as an inhibitor of retinal neovascularization was studied in an *in vivo* rat model of retinopathy of prematurity, as described by Xavier and Dorey, (1994) Invest. Ophthalmol Vis. Sci. 35, 8. The effects of *in vivo* local and parenteral administration in terms of adverse toxic or immunological reactions such as uveitis, endophthalmitis, and serum sickness were determined, as well as the effective time period for heparinase administration with respect to the neonatal day of exposure to hypoxia.

Materials and Methods

Oxycycler: An oxycycler was used to maintain animals under controlled hypoxia. The set up monitors the inflow of oxygen and nitrogen, and senses the concentration of oxygen in the incubation chamber which houses the animals. It

can be attached to a computer keyboard and monitor to record the data. The neonatal rats with their mother were housed in plexiglass boxes in an incubation chamber which has an oxygen sensor
5 connected to the oxycycler.

Intravitreal injections: The intravitreal route of administration was used since it is the closest to local uniform delivery to the entire retina. The mice were anesthetized by
10 methoxyfluorane inhalation, which has a very low mortality rate. The technique of intravitreal injection involves teasing the eyelids open with a fine pair of scissors, instilling local anesthetic eye drops, holding the eyelids open with a
15 retractor or forceps, placing a drop of methylcellulose (Goniosol™) eye drops to view the inside of the eye, and using a surgical microscope with co-axial light to visualize the inside of the eye, including the posterior capsule of the lens,
20 the vitreous cavity and the retina. A Hamilton syringe with a 32G needle was used to inject intravitreally going through the coats of the eye about 3 mm behind the limbus.

Dissection, Staining and Scoring of
25 *neovascularization:* After 18 days of interrupted oxygen exposure, the animals were sacrificed and the eyes enucleated and fixed in 0.4 M Cacodylate in 4% Formaldehyde, then were stored in Tris buffer. The eyes were then dissected and the
30 retinas were isolated. The retinal vasculature was stained using ADPase histochemistry; the retinas were either flat mounted, or were embedded and sectioned to study the vessels. Various parameters, including the avascular area and
35 neovascularization, were measured using image analysis by a processor attached to a Photomicroscope. Scoring was done on flat mounts,

depending upon the number of tufts and the number of quadrants involved.

Heparinase I was used in this study at a concentration of 4 μ l into the central vitreous area.

5 **Animals:** Timed pregnant Sprague Dawley albino rats were bought from Taconic. The newborn rats were randomized, one set placed with their mother in the high oxygen chamber, and the other
10 set exposed only to room air. The pattern of hypoxia used in the former set of rats was intermittent exposure to room air (20% oxygen) for 30 minutes every day, with a gradual increase to
15 80% oxygen over 3 hours. This pattern was followed for 11 days, starting the day after birth. The animals were then left in room air for another 6 days. On neonatal day 11, the animals from both the groups were randomized to receive intravitreal
20 injections of heparinase or vehicle or to receive no injections. They were sacrificed on day 18 and their eyes enucleated, fixed, dissected, stained, mounted and studied using image analysis.

Results:

25 Neither the heparinase injected nor the vehicle injected eyes showed any signs of inflammation in any of the eyes. This is in contrast to some of the other inhibitors of neovascularization which have shown tremendous inflammation upon intravitreal injection. Based on
30 comparison of the treated eyes by dissection, staining and mounting, heparinase does not seem to affect the integrity of the retina, nor does the enzyme affect the normally developing vasculature of the retina.

35 Preparation of Pharmaceutical Compositions

In vitro and *in vivo*, heparinases I and III are among the most potent and effective

angiogenesis inhibitors known to date. *In vivo*, about 4 μ g of heparinases I or III (105 and 54 pmol, respectively) in methyl cellulose discs cause 100% inhibition of embryonic neovascularization, resulting in large avascular zones; *in vitro*, heparinases I and III significantly inhibit bFGF mediated bovine capillary endothelial proliferation, with IC₅₀ values of 6 and 21 nM respectively.

10 The most important attribute of any therapeutic modality is its selectivity towards the cell type and tissues that it is meant to target. The histology studies on the CAM assay demonstrate that the quiescent non-proliferating endothelial as well as other cell types, such as the pericytes and the fibroblasts, are unaffected by heparinase. Angiogenesis inhibition by heparinases is not due to non-specific heparin degradation, as heparinase II does not affect proliferating endothelial cells or angiogenesis *in vivo*.

20 As noted above, several types of heparinase can be used as described herein to inhibit angiogenesis. The effective dosage will depend on the purity and origin of the heparinase, as well as the type, for example, heparinase I versus heparinase II. The preferred enzymes are heparinase I and heparinase III from *F. heparinum*. Pharmaceutical compositions are prepared using heparinase as the active agent to inhibit angiogenesis based on the specific application. Application is either topical or localized. For topical application, the purified heparinase is combined with a carrier so that an effective dosage is delivered, based on the desired activity, i.e. ranging from an effective dosage, for example, of 1 and 4 μ g heparinase (with 100% inhibition of angiogenesis at between 3 and 4 μ g) to prevent

localized angiogenesis to between 10 and 100 nM to inhibit capillary endothelial cell proliferation, with 100% inhibition at approximately 80 nM heparinase I or III. A topical heparinase composition is applied to the skin for treatment of diseases such as psoriasis. The carrier may be in the form of an ointment, cream, gel, paste, foam, aerosol, suppository, pad or gelled stick.

Neovascularization is known to be important in the pathogenesis of many potentially blinding disorders of the eye like diabetic retinopathy, age related macular degeneration, retinal branch vein occlusion and retinopathy of prematurity (ROP). A topical heparinase composition for treatment of some of the eye disorders discussed above consists of an effective amount of heparinase in an acceptable ophthalmic excipient such as buffered saline, mineral oil, vegetable oils such as corn or arachis oil, petroleum jelly, Miglyol 182, alcohol solutions, or liposomes or liposome-like products. Any of these compositions may also include preservatives, antioxidants, antibiotics, immunosuppressants, and other biologically or pharmaceutically effective agents which do not exert a detrimental effect on the heparinase.

Heparinase compositions for local or regional administration, for example, into a tumor, will generally include an inert diluent, and will be administered in an amount effective to inhibit angiogenesis, as noted above. Solutions or suspensions used for parenteral, intradermal, subcutaneous, or topical application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens;

antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parental preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. Commercially available heparinase preparation come either lyophilized or in sterile water or buffer, but are rarely of the purity that is desired for this application.

For directed internal topical applications, for example for treatment of ulcers or hemorrhoids, or other lesions of mucosal membranes, the heparinase composition may be in the form of tablets or capsules, which can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; or a glidant such as colloidal silicon dioxide. When the dosage unit form is a capsule, it can contain, in addition to material of the above type, a liquid carrier such as a fatty oil. In addition, dosage unit forms can contain various other materials which modify the physical form of the dosage unit, for example, coatings of sugar, shellac, or other enteric agents.

In a preferred form, the heparinase is administered in combination with a biodegradable, biocompatible polymeric implant which releases the heparinase over a controlled period of time at a selected site. Examples of preferred polymeric materials include polyanhydrides, polyorthoesters,

polyglycolic acid, polylactic acid, cellulose acetate, methyl cellulose, polyethylene vinyl acetate, and copolymers and blends thereof, and gels formed of materials such as a polysaccharide
5 such as alginate or heparin, which is bound by heparinase in the absence of calcium. In the latter case, the heparinase is released under physiological conditions. Heparinase can also be administered at a local site by infusion pump, for
10 example, of the type used for delivering insulin or chemotherapy to specific organs or tumors.

The compositions are administered in an effective amount to inhibit angiogenesis, as demonstrated by reference to the examples provided
15 above. Optimization of the mode and time of delivery is made by those skilled in the art of treatment of the disorder being treated, and will vary from patient to patient.

We claim.

1. A composition inhibiting angiogenesis comprising an effective amount of a heparinase in a pharmaceutically acceptable carrier to inhibit angiogenesis at a selected site in a non-heparinized patient in need of treatment thereof.

2. The composition of claim 1 wherein the heparinase is in a carrier delivering to a site where angiogenesis is to be inhibited between approximately one and four μg heparinase or a concentration of between 10 and 100 nM heparinase.

3. The composition of claim 1 wherein the heparinase is in an acceptable carrier for topical application.

4. The composition of claim 3 wherein the heparinase is in an acceptable ophthalmic carrier.

5. The composition of claim 3 wherein the heparinase is in a pharmaceutically acceptable carrier for topical application to the skin.

6. The composition of claim 1 wherein the heparinase is in a pharmaceutically acceptable carrier for parenteral or enteral administration.

7. The composition of claim 1 wherein the heparinase is in a biodegradable, biocompatible polymeric delivery device.

8. The composition of claim 1 wherein the heparinase is derived from *Flavobacterium heparinum*.

9. The composition of claim 8 wherein the heparinase is selected from the group consisting of heparinase I, heparinase II and heparinase III.

10. A method for inhibiting angiogenesis comprising

administering to a non-heparinized patient in need of treatment thereof an effective amount of a heparinase in a pharmaceutically acceptable

carrier to a site where inhibition of angiogenesis is desired.

11. The method of claim 10 wherein the site is a tumor.

12. The method of claim 10 further comprising administering the heparinase topically in a pharmaceutical vehicle suitable for topical application.

13. The method of claim 10 further comprising providing the heparinase in a biodegradable, biocompatible polymeric delivery device.

14. The method of claim 10 further comprising providing the heparinase in a pharmaceutically acceptable vehicle for injection.

15. The method of claim 10 further comprising administering the heparinase in a pharmaceutically acceptable vehicle for topical application to the eye.

16. The method of claim 10 wherein an effective dose at the site where angiogenesis is to be inhibited is between approximately one and four μg heparinase or a concentration of between 10 and 100 nM heparinase.

17. The method of claim 10 wherein the effective dose is a dose effective in diminishing the symptoms of psoriasis.

18. The method of claim 10 wherein the effective dose is a dose effective in diminishing the number of blood vessels growing into a tumor.

19. The method of claim 10 wherein the effective dose is a dose effective in diminishing the symptoms of eye diseases characterized by abnormal neovascularization.

20. The method of claim 10 wherein the heparinase is derived from *Flavobacterium heparinum*.

21. The method of claim 20 wherein the heparinase is selected from the group consisting of heparinase I and heparinase III.

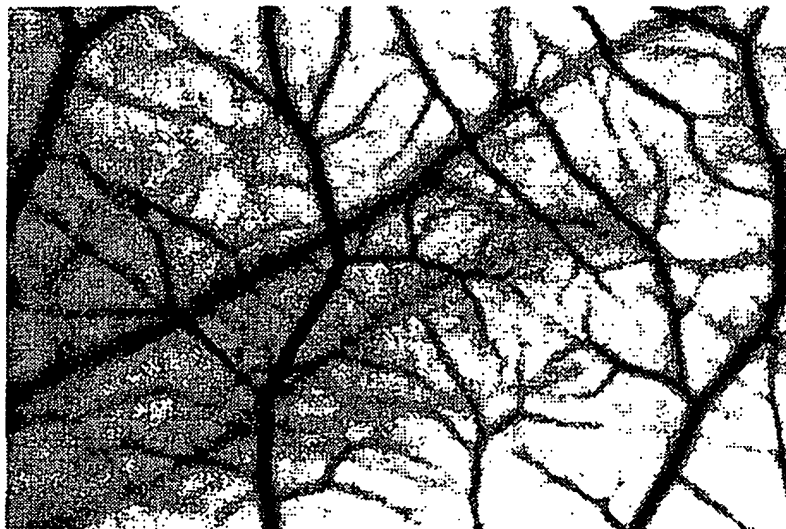


FIGURE 1a



FIGURE 1b

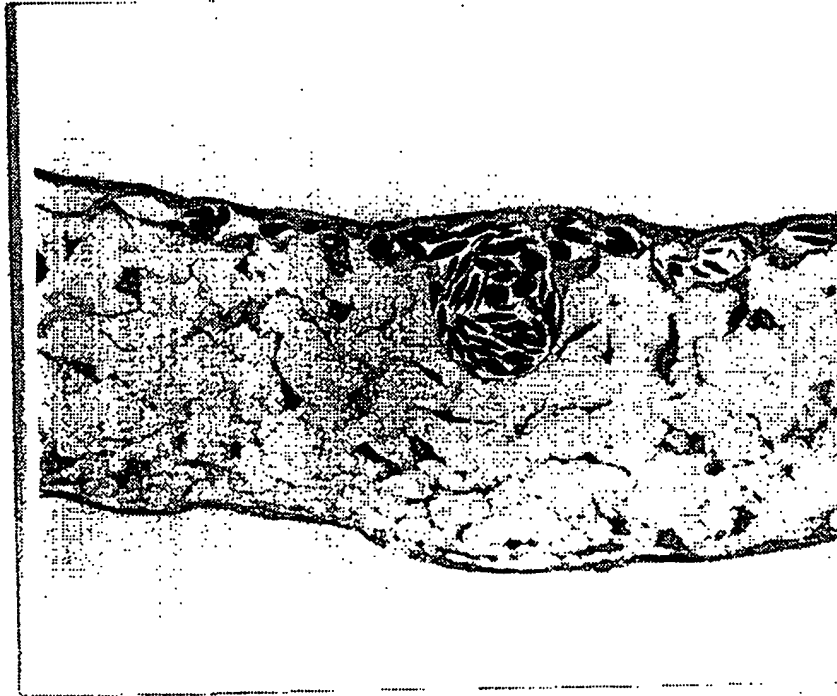


FIGURE 1c

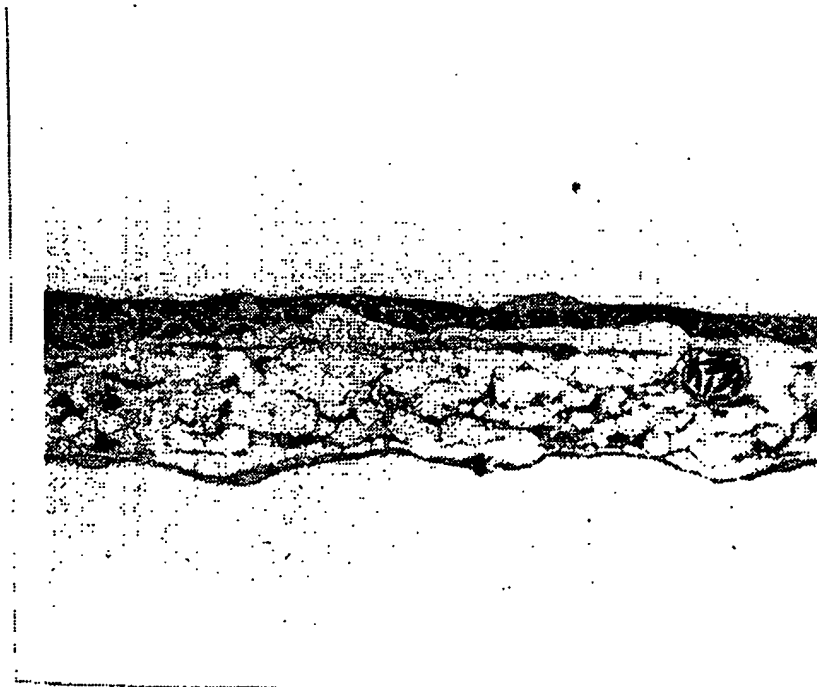


FIGURE 1d

3/4

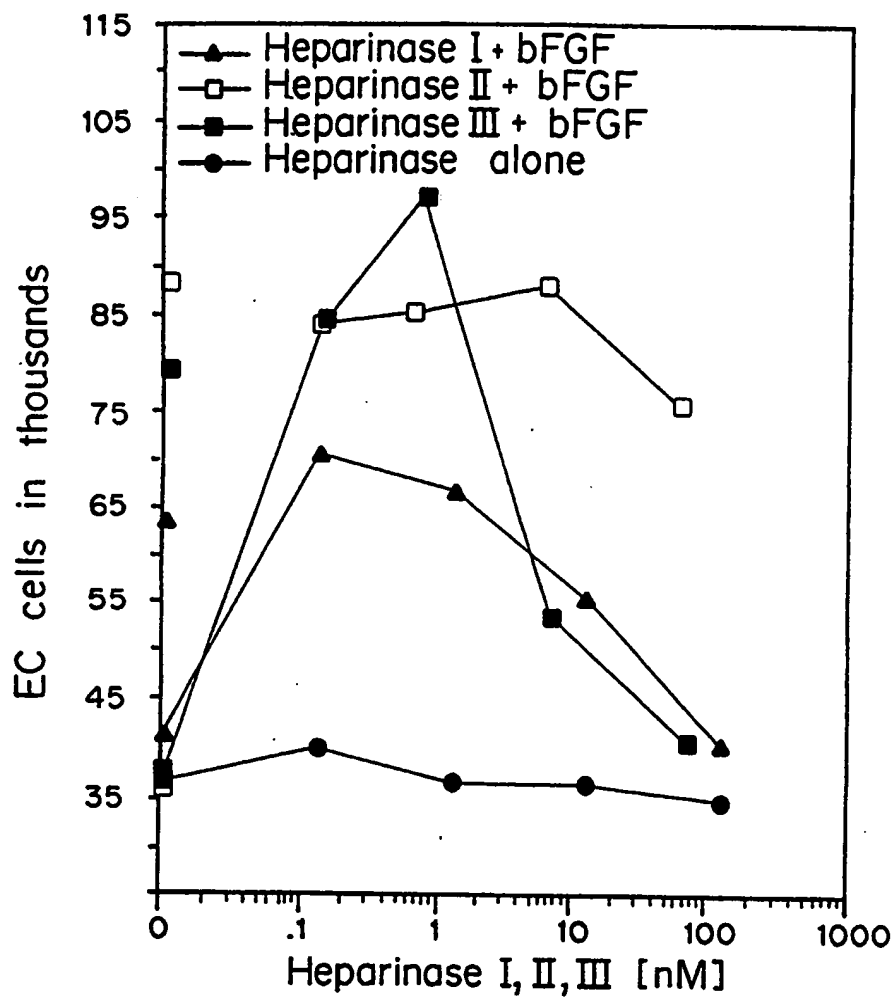


FIG. 2

4/4

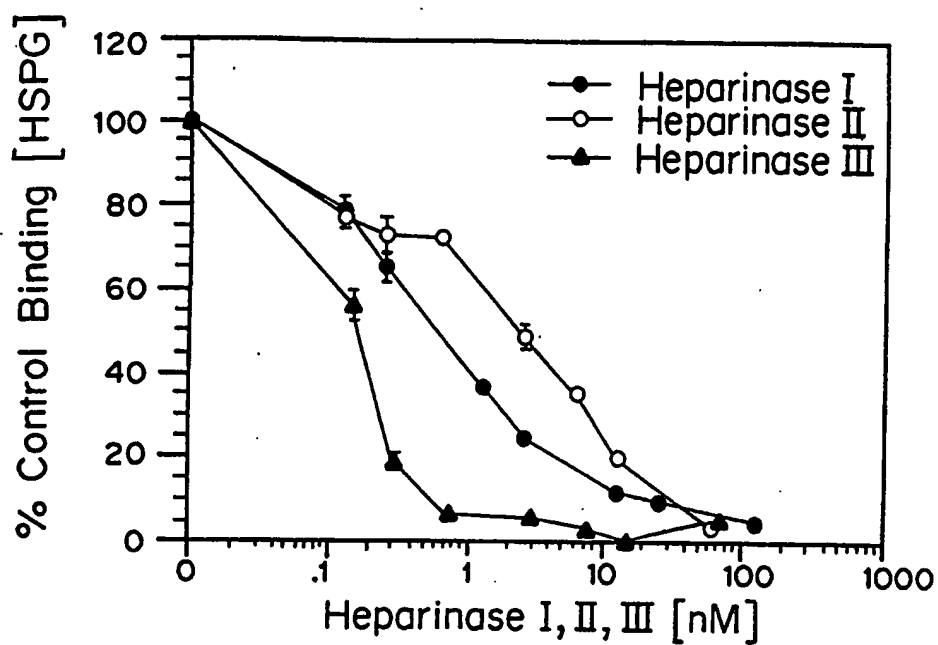


FIG. 3a

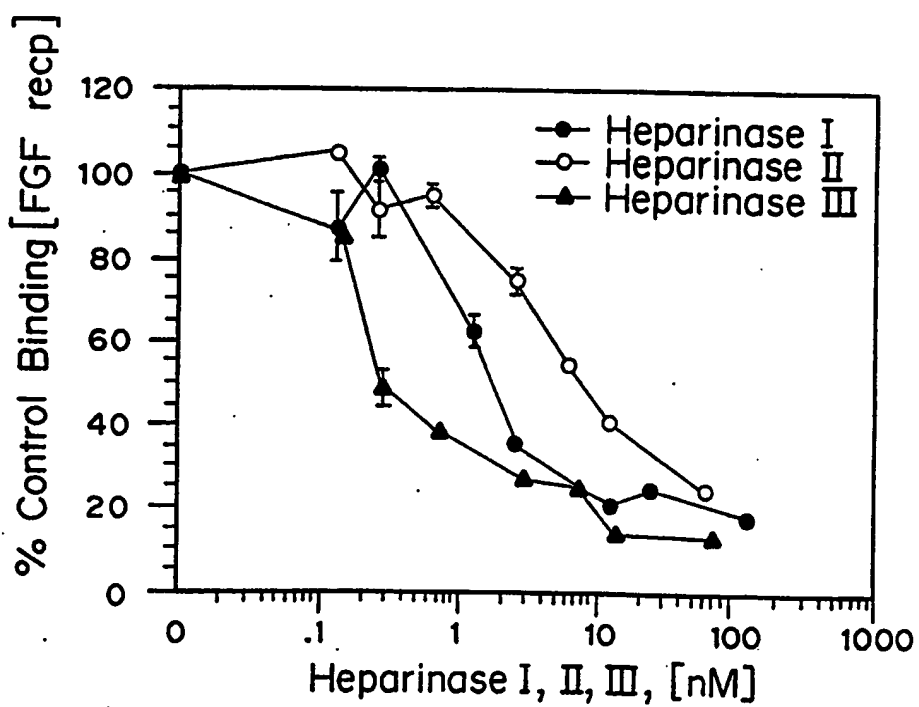


FIG. 3b

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/13343

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/51

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DAVID PUETT ET AL. 'ADVANCES IN GENE TECHNOLOGY: MOLECULAR BIOLOGY OF THE ENDOCRINE SYSTEM' 1986, CAMBRIDGE UNIVERSITY PRESS, CAMBRIDGE ET AL. HEPARINASE TREATMENT OF ENDOTHELIAL CELLS PREVENTS RESPONSE TO HEPATOMA-DERIVED GROWTH FACTOR by DANIEL T. CONNOLLY ET AL. see page 110 - page 111 ---	1,8
X,P	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol.91, February 1994, WASHINGTON US pages 1524 - 1528 RAM SASISEKHARAN ET AL. 'HEPARINASE INHIBITS NEOVASCULARIZATION.' see the whole document --- -/--	1-21

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

13 March 1995

Date of mailing of the international search report

20 -03- 1995

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Rempp, G

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/13343

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,O 394 971 (KABIVITRUM) 31 October 1990 -----	

INTERNATIONAL SEARCH REPORT

II. International application No.

PCT/US 94/ 13343

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claims 10-21 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

...ation on patent family members

PCT/US 94/13343

Form PCT/ISA/210 (patent family annex) (July 1992)